

THE ASSOCIATION OF RHODAMINE - LABELLED  $\alpha$ -ACTININ WITH  
ACTIN BUNDLES IN DEMEMBRANATED CELLS

BENJAMIN GEIGER

Department of Chemical Immunology, The Weizmann Institute of Science,  
76100 Rehovot, Israel

ABSTRACT

Rhodamine-labelled  $\alpha$ -actinin was specifically bound to actin containing filament bundles of demembrated fibroblasts, and was particularly associated with their termini. Optimal binding of rhodamine- $\alpha$ -actinin occurred at pH 6.0 - 6.2 and could be abolished by the addition of unlabeled  $\alpha$ -actinin or myosin subfragment 1. The spatial relationships between the decorating  $\alpha$ -actinin and several actin associated proteins was determined by double fluorescence microscopy.

INTRODUCTION

Much progress has been made in recent years in the molecular characterization of the contractile system in non-muscle cells. This was largely due to the isolation and identification of a variety of mechanochemical proteins, and to the development of immunocytochemical techniques that may be used to analyse the intracellular distributions of these proteins. In addition to actin, which is the major component of cellular microfilaments, non muscle cells contain several proteins that are associated with actin, including myosin, tropomyosin, filamin and  $\alpha$ -actinin (Pollard and Weihing, 1974; Korn, 1978). All these proteins bind to isolated F-actin (Pollard and Weihing, 1974; Wang and Singer, 1977; Holmes et al. 1971) and their distributions within cells are related to that of actin in a pattern specific for each of these proteins as determined by fluorescence microscopy (Fujiwara and Pollard, 1976; Lazarides and Burridge, 1975; Wang et al. 1975; Lazarides, 1976; Heggeness et al., 1977)

It is still unknown how this complex, yet highly organized, arrangement of cytoskeletal proteins is generated. One approach proposed for studying the assembly of the microfilament-associated system is the microinjection of fluorophore-conjugated mechanochemical proteins and the microscopic examination of their subsequent intracellular distributions. This approach was found feasible for several proteins including actin (Kreis et al., 1979),  $\alpha$ -actinin (Feramisco, 1979) and vinculin (130K protein) (Burridge and Feramisco, 1980). All three proteins were incorporated into the microfilament-associated system in a pattern similar to that of the respective, native proteins. It is, however, still unclear whether the incorpo-

ration of these cytoskeletal proteins into the microfilament system is an active, dynamic cellular process, that can occur only in the intact viable cells, or whether fully developed stress fibers can passively bind these proteins in a highly specific manner. It is here demonstrated that rhodamine-conjugated  $\alpha$ -actinin binds to the stress fibers and in particular to their termini in non viable, demembrated fibroblasts. Similarly treated monolayers of kidney epithelial cells bind  $\alpha$ -actinin in limited areas along their intercellular junctions.

#### MATERIALS AND METHODS

Chick gizzard fibroblasts were prepared from 10 day chick embryos, and maintained in culture in DME-medium containing 10% Fetal Calf serum. Kidney epithelial line MDCK was obtained from Dr. D. Louvard, EMBL, Heidelberg. Cells were permeabilized by a 2 minute treatment with 50 mM MES buffer, 5 mM  $MgCl_2$ , 3 mM EGTA, 0.8%  $\alpha$ -octylglucoside pH 6.1. Rhodamine-labeled  $\alpha$ -actinin was prepared as described (Feramisco, 1979), and contained an average of 3-5 fluorophores/molecule. Moysin subfragment 1 (S1) was prepared according to Weeds and Pope (1977). Antibodies to the various actin associated proteins were prepared as described previously (Geiger and Singer, 1979; Geiger, 1980). Fluorescent microscopy was performed with Zeiss Photoscope III equipped with filter sets for selective observation of rhodamine and fluorescein.

#### RESULTS AND DISCUSSION

The pattern of decoration of the demembrated chicken fibroblasts by Rh- $\alpha$ -actinin is monstrated in Figure 1. The major sites to which the labelled  $\alpha$ -actinin bound were elongated patches at the bottom of the cells (see arrow in Fig. 1A). When observed with interference-reflection optics, these arrowhead-shaped patches (Fig. 1B) coincided with the interference-reflection dark images of focal adhesion plaques (Fig. 1C). The edge of the peripheral lamellipodium was often diffusely labelled by the Rh- $\alpha$ -actinin (Fig. 1A, arrowhead). In more central areas,  $\alpha$ -actinin was bound in a typical striated pattern to stress fibers and broad elongated sheets (Fig. 1D, Fig. 3I). Many of the spreading cells showed intensely decorated dots around the nucleus in an arrangement similar to that described previously for cells immunolabelled with anti- $\alpha$ -actinin antibodies (Lazarides and Burridge, 1975; Lazarides, 1976).

When a 10 fold excess of unlabelled  $\alpha$ -actinin was added to the rhodamine labelled protein, the fluorescent labelling was almost completely abolished (Fig. 1E and F). Overall, the pattern of  $\alpha$ -actinin decoration obtained here was similar to that obtained by immunolabelling after similar treatments (see Fig. 1G) or after fixation and Triton permeabilization as published (Lazarides and Burridge, 1975; Lazarides, 1976; Feramisco, 1979). The specificity of binding of the fluorescent  $\alpha$ -actinin to stress fibers and their termini was further indicated by the absence of labelling of these areas non specifically by rhodamine conjugated BSA or goat immunoglobulin.

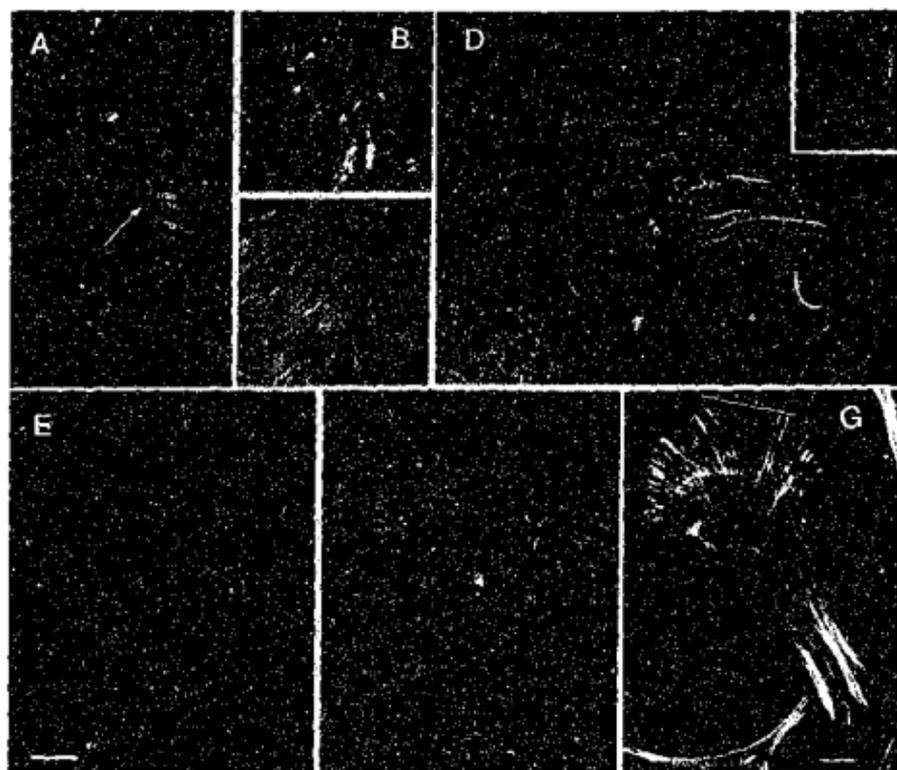


Figure 1. Decoration of demembrated chicken gizzard fibroblasts with rhodamine conjugated  $\alpha$ -actinin. Cells were incubated for 2 minute with 0.8%  $\alpha$ -octyl-glucoside in 50 mM MES buffer 3mM EGTA, 5 mM  $MgCl_2$  pH 6.1 and then incubated for 10 minutes with 40  $\mu$ g/ml rhodamine- $\alpha$ -actinin in the same buffer.

A and D: Cells decorated with rh- $\alpha$ -actinin, note the extensive labelling of the elongated plaques (arrows) the spotted fibers and sheets and labelling of the lamellipodium (arrowhead).

B and C: Fluorescent decoration with rh- $\alpha$ -actinin and the corresponding area, observed with interference reflection optics. Note the coincidence of intensely labelled sites with cell-substrate contacts (interference-reflection-dark areas). Arrowhead points to the same area. E and F: Inhibition of the decoration with rh- $\alpha$ -actinin by 10 fold excess of the unlabelled protein (E). The same area observed by phase optics is shown in F. G: Demembrated fibroblast, labelled with rabbit anti  $\alpha$ -actinin and rh-goat anti rabbit IgG.

Bars indicate 10  $\mu$ .

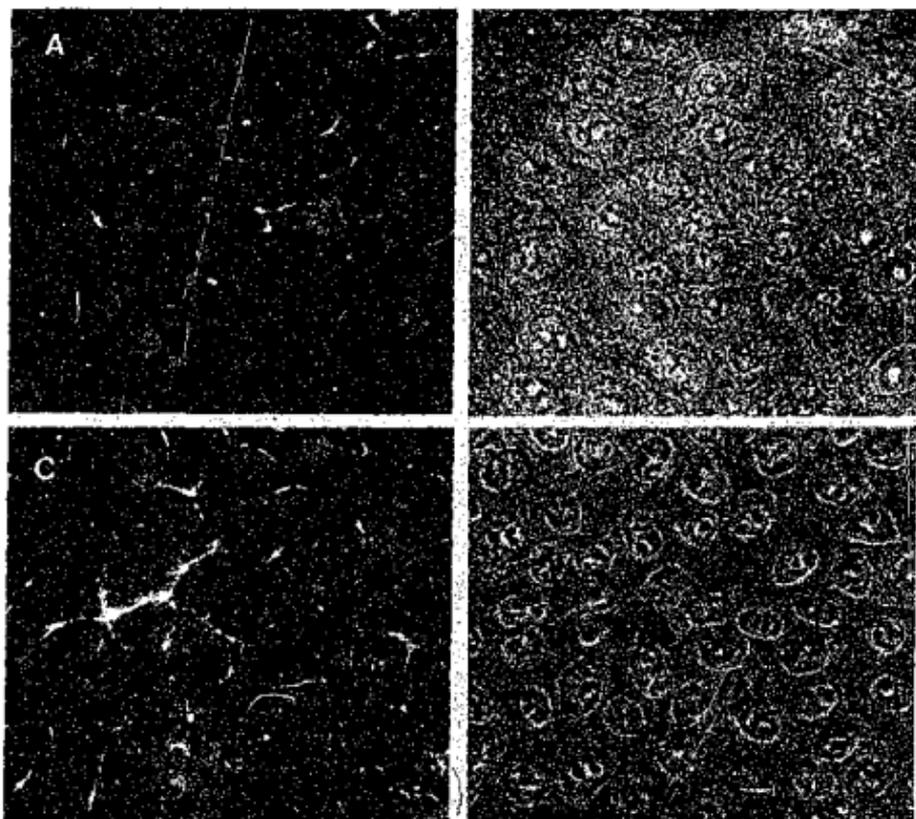


Figure 2. A: Demembrated MDCK cells, decorated with rh- $\alpha$ -actinin. Notice the association of the fluorescent protein with the intercellular junction. Focus was at the upper portion of the cell where the tight junction is located. B: Phase micrograph of the same area shown in A. C: Immunofluorescent labelling of MDCK cells with rabbit anti  $\alpha$ -actinin and rh-goat anti rabbit IgG. Notice the similarity of the immunolabelling pattern (C) to that obtained by rh- $\alpha$ -actinin binding (A). D: Phase micrograph of the same area shown in C. Arrows point to the same tight junction areas in the pairs A-B and C-D. Bar indicate 10  $\mu$ .

Fluorescent S<sub>1</sub> which decorates the stress fibers gives only poor labelling of the focal adhesion plaques.

A different cell type in which the decoration with Rh- $\alpha$ -actinin was attempted was a kidney epithelial cell line, MDCK. These cells

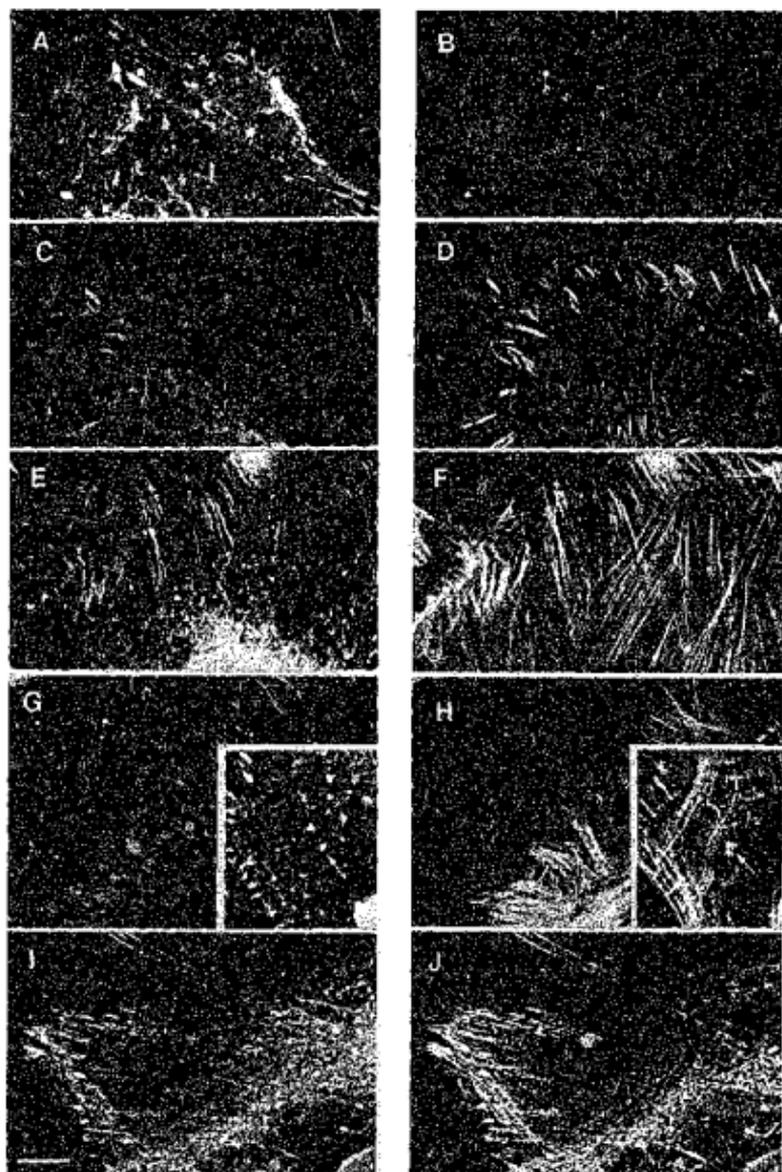
are devoid of visible stress fibers and the binding of Rh- $\alpha$ -actinin after demembration was mainly confined to an area along the peripheral tight junctions (see matching arrows in Fig. 2A and B).

This is also the area where most of the cellular  $\alpha$ -actinin is concentrated, (Louvard, 1980) as visualized here by indirect immunofluorescence (Fig. 2C and D).

The spatial relationships between the decorating  $\alpha$ -actinin and other constituents of the microfilament-associated system were determined by double fluorescent labelling. Cells were decorated with Rh- $\alpha$ -actinin, fixed and immunolabelled indirectly for actin, vinculin, tropomyosin, filamin and  $\alpha$ -actinin. The pairs of rhodamine-fluorescein labelled cells for  $\alpha$ -actinin and each of these proteins, respectively (shown in Fig. 3) indicated that the two were closely related. Actin antibodies (Fig. 3B) labelled the entire length of the stress fibers including the termini where  $\alpha$ -actinin was abundant (Fig. 3A). Vinculin was associated with  $\alpha$ -actinin in those terminal areas exclusively (Fig. 3C and D). This is in line with the reported cellular distribution of vinculin and its association with focal adhesion plaques (Geiger, 1980). The close association of vinculin with both the cell membrane and with  $\alpha$ -actinin was demonstrated by immunofluorescence and immunoelectron microscopy in several systems including cultured fibroblasts, intestinal epithelium, smooth muscle cells and cardiac muscle (Geiger, 1980; Geiger et al., 1980). Filamin labelling (Fig. 3F) was similar to that of actin, and was similarly related to the Rh- $\alpha$ -actinin (Fig. 3E). Tropomyosin labelling was also associated with stress fibers, (Fig. 3H) however it was largely depleted from the termini of these bundles where Rh- $\alpha$ -actinin was enriched (Fig. 3G). The labelling patterns obtained by decoration with Rh- $\alpha$ -actinin and subsequently by immunolabelling of the same cells for that protein with fluorescein-conjugated antibodies (Figs. 3I and J, respectively) were almost identical. It is noteworthy that apparently all the sites that contained endogenous  $\alpha$ -actinin also bound that protein when added exogenously to the demembrated cells.

Attempts to inhibit the binding of Rh- $\alpha$ -actinin to the cells by antibodies against actin, myosin,  $\alpha$ -actinin, filamin and vinculin were not successful. However, the addition of myosin subfragment 1 was an effective inhibitor of  $\alpha$ -actinin binding. The Rh- $\alpha$ -actinin and S1 were mixed at 3 different molar ratios (5:1, 1:1 and 1:5) keeping the  $\alpha$ -actinin concentration constant: 400  $\mu$ g/ml in 50 mM MES, 5mM MgCl<sub>2</sub>, 3 mM EGTA at pH of 6.1 or 7.0. At pH 7.0, a marked inhibition of  $\alpha$ -actinin decoration was observed microscopically at a 1:1 ratio of the two cytoskeletal components, and the inhibition was complete when a 5 fold excess of S1 was used. At pH 6.1 the inhibition by S1 was less effective, and was partial even at a 5:1 ratio of S1 to  $\alpha$ -actinin. It is suggested that the two components can bind to cellular actin probably at the same or closely related sites. The pH optima for the binding of these two components to actin in situ were different though the physiological significance of this difference is not clear.

The use of directly fluoresceinated cytoskeletal proteins may be useful in two aspects related to the structure and function of the



cellular contractile system: (a) In an analogy to the binding of heavy meromyosin or S1 to actin, a procedure that proved most helpful for the identification of F-actin filaments and the determination of their polarity, (Ishikawa et al., 1969; Mooseker and Tilney, 1975) the decoration of intact microfilaments with other actin associated proteins such as  $\alpha$ -actinin may help define functional domains in the actin-associated system. Moreover, modification of the conditions used for decoration such as pH, ionic strength, divalent ions and the presence of other related proteins, may help define the balanced physiological interactions in the actin associated system. (b) The second aspect of this study is related to the formation and maintenance of the complex microfilament associated system. It indicates that  $\alpha$ -actinin may be passively incorporated into specific regions along mature stress fibers, without the requirement for an active and dynamic participation of the cells. This notion is important for the interpretation of results obtained by microinjections of fluorescently-labelled proteins including  $\alpha$ -actinin into living cells.

#### ACKNOWLEDGEMENT

I wish to express my gratitude to Mrs. H. Kupfer for her excellent technical assistance.

This study was supported by a grant from the Muscular Dystrophy Association.

#### REFERENCES

- Burridge, K. and Feramisco, J.R. (1980) Microinjection and localization of a 130 K protein in living fibroblasts: a relationship to actin and fibronectin. *Cell* 19, 587-595.  
 Feramisco, J.R. (1979) Microinjection of fluorescently labeled  $\alpha$ -actinin into living fibroblasts. *Proc. Natl. Acad. Sci. USA*, 76, 3967-3971.

---

Figure 3. Double fluorescent labelling with rh- $\alpha$ -actinin and antibodies to several microfilament associated proteins in chicken gizzard cells. The cells were demembranated as above and incubated with rh- $\alpha$ -actinin. The monolayer was then rinsed, fixed with paraformaldehyde for 30 min and immunolabelled indirectly with rabbit antibodies to the various cytoskeletal components and fluorescein-conjugated goat anti rabbit-IgG. A,C,E,G,I : Demembranated cells decorated with rh- $\alpha$ -actinin. B: The same cell as in A, fixed and immunolabelled indirectly with antibodies (IgG fraction) to chick muscle actin and fluorescein conjugated goat anti-rabbit IgG. D: The same cell as in C, immunolabelled for vinculin. F: The same cell as in E, immunolabelled for filamin. H: The same cell as in G, immunolabelled for tropomyosin. The bright  $\alpha$ -actinin dots in the insert in G fit into the vertices of the polygonal tropomyosin-containing structures. J: The same cell as in I, immunolabelled for  $\alpha$ -actinin. The bar indicates 10  $\mu$ .

- Fujiwara, K. and Pollard, T.D. (1976) Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells. *J. Cell Biol.* 71, 848-875.
- Geiger, B. (1979) A 130K protein from chicken gizzard: its localization at the terminal of microfilament bundles in cultured chicken cells. *Cell* 18, 193-205.
- Geiger, B. and Singer, S.J. (1979) The participation of  $\alpha$ -actinin in the capping of cell-membrane components. *Cell* 16, 213-222.
- Geiger, B., Tokuyasu, K.T., Dutton, A.H. and Singer, S.J. (1980) Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA.* 77, 4127-4131.
- Heggeness, M.H., Wang, K. and Singer, S.J. (1977) Intracellular distributions of mechanochemical proteins in cultured cells. *Proc. Natl. Acad. Sci. USA.* 74, 3883-3887.
- Holmes, G.R., Gall, D.E. and Suzuki, A. (1971) Effect of  $\alpha$ -actinin on actin viscosity. *Biochim. Biophys. Acta* 253, 240-253.
- Ishikawa, H., Bischoff, R. and Holzer, H. (1969) Formation of arrow-head complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* 43, 312-328.
- Korn, E.D. (1978) Biochemistry of actomyosin-dependent cell motility (a review) *Proc. Natl. Acad. Sci. USA.* 75, 588-599.
- Kreis, T.E., Winterhalter, K.H. and Birchmeier, W. (1979) In vivo distribution and turnover of fluorescently labelled actin microinjected into human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76, 3814-3818.
- Lazarides, E. (1976) Actin,  $\alpha$ -actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. *J. Cell Biol.* 68, 202-219.
- Lazarides, E. and Burridge, K. (1975)  $\alpha$ -actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell* 6, 289-298.
- Louvard, D. (1980) Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77, 4132-4136.
- Mooseker, M.S. and Tilney, L.G. (1975) The organization of an actin filament-membrane complex: filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.* 67, 725-743.
- Pollard, T.D. and Weihing, R.R. (1974) Actin and myosin and cell movement. *CRC Crit. Rev. Biochem.* 2, 1-65.
- Wang, K., Ash, F. and Singer, S.J. (1975) Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 72, 4483-4486.
- Wang, K. and Singer S.J. (1977) Interaction of filamin with F-actin in solution. *Proc. Natl. Acad. Sci. USA.* 74, 2021-2025.
- Weeds, A.G. and Pope, B. (1977) Studies on the chymotryptic digestion of myosin. Effect of divalent cations on proteolytic susceptibility. *J. Mol. Biol.* 111, 129-157.